

# Exosome-mediated miR-1290 regulation of SLU7 affects hepatocellular carcinoma process

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## Research article

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# Abstract

**Objective:** Liver cancer, as a very common malignant tumor in clinic, has a high incidence rate and a poor prognosis. Understanding the relationship of exosomes miR-1290 and SLU7 with liver cancer may be a breakthrough in the diagnosis and treatment of liver cancer in the future.

**Methods:** Altogether 64 patients with liver cancer and 58 healthy people in Shandong Cancer Hospital affiliated to Shandong University were collected, and liver cancer cells were purchased. qPCR was used to detect the mRNA levels of miR-1290 and SLU7. Western blot was used to detect the relative expression of SLU7 protein, and the effect of exosomes miR-1290 and SLU7 on the biological behavior of liver cancer cells was analyzed. Double fluorescein reporter was used to verify the targeted relationship between miR-1290 and SLU7.

**Results:** miR-1290 was highly expressed in liver cancer ( $P < 0.050$ ) and SLU7 was poorly expressed in liver cancer ( $P < 0.050$ ). Exosomes miR-1290 could inhibit the relative expression of SLU7 protein ( $p < 0.050$ ), promote the proliferation, invasion, migration and epithelial-mesenchymal transformation of liver cancer cells, and reduce the ability of apoptosis ( $P < 0.050$ ). Double luciferase report and rescue experiment confirmed that miR-1290 and SLU7 had targeted relationship ( $P < 0.050$ ).

**Conclusion:** Exosomes miR-1290 may be a potential target for the diagnosis and treatment of liver cancer in the future through targeting inhibition of SLU7 in the occurrence and development of liver cancer.

## Introduction

Hepatocellular carcinoma is a very common malignant tumor worldwide, with a high incidence rate [1]. According to statistics, the incidence of liver cancer is about 400,000 cases worldwide at present, and it is increasing year by year in recent years [2, 3]. Moreover, the symptoms of early liver cancer are usually nonspecific and easy to be ignored or mismanaged by patients. Once diagnosed, the disease has developed to the middle and late stages [4]. At this stage, it is difficult to cure liver cancer and the prognosis of patients is not optimistic. Statistics show that 745,000 patients die of liver cancer every year [5]. At present, the pathogenesis of liver cancer is not yet clear. Studies believed that the pathogenesis of liver cancer is a complex process with multiple factors and steps, and is affected by environment and diet [6]. With the deepening of research, more and more scholars have focused on miRNA.

miRNA contains about 22–25 nucleotides and can target the 3'- untranslated region (3'-UTR) of its target gene mRNA according to the strict complementary nature of the base pairs to inhibit the translation of its target gene or directly induce mRNA degradation. Currently, miRNA has been proved to be involved in the occurrence and development of many tumors [7–9]. Among them, miR-1290 is a newly discovered miRNA, and previous studies have shown that it is closely related to the occurrence of colon cancer [10]. More studies have claimed that miR-1290 can promote proliferation of lung adenocarcinoma cells through SOCS4 [11].

However, SLU7 protein is a splicing protein closely related to liver function. The research by Wang et al. [12] confirmed that improving SLU7 protein in mice can alleviate liver injury, while the research by Jimenez [13] et al. showed that SLU7 is crucial to maintain liver differentiation.

At present, the relationship between miR-1290 and liver cancer is not clear, and studies on miR-1290 and SLU7 in exosomes are even rarer. Facing the increasingly high incidence of liver cancer diseases, this experiment will explore the molecular mechanism of miR-1290 and SLU7 in exosomes on liver cancer, providing reliable reference and guidance for clinical diagnosis and treatment of liver cancer in the future.

## **Materials And Methods**

### **1. General information**

Sixty-four patients with liver cancer admitted to Shandong Cancer Hospital affiliated to Shandong University from July 2015 to July 2016 were selected as the study group, and another 58 healthy people were selected as the control group for prospective analysis. This experiment has been approved by the Ethics Committee of Shandong Cancer Hospital affiliated to Shandong University. All the above research subjects have signed informed consent forms by themselves or their immediate family members. There was no statistical difference between the two groups in general data such as age, gender and BMI ( $P > 0.050$ ).

### **2. Inclusion and exclusion criteria**

The inclusion criteria of the study group were as follows: patients were confirmed as primary liver cancer by biopsy of pathology department of Shandong Cancer Hospital affiliated to Shandong University; patients aged 30~60 years; patients with complete case data. Exclusion criteria: patients with other tumors; patients with other cardiovascular and cerebrovascular diseases; patients with other infectious diseases; patients with other autoimmune dysfunction; patients with major organ failure (except the lung); patients with mental disorders; patients received other antibiotics, chemotherapy, radiotherapy and surgery within half a year before admission; patients with low treatment compliance; pregnant and lactating patients; patients transferred to hospital; patients with an estimated survival period  $\leq 1$  month. The inclusion criteria of the control group: people with normal physical examination in Shandong Cancer Hospital affiliated to Shandong University; all physical examination results were normal; people with complete cases and no previous major medical history; people aged 30~60 years.

### **3. Cell data**

Human hepatoma cells HepG2, Huh-7, BEL-7402, SMMC-7721 and human normal hepatocyte HL-7702 were all purchased from ATCC cell bank. The cells were cultured in DMEM medium (10% fetal bovine serum +10% penicillin-streptomycin) at 37°C with 5%CO<sub>2</sub>, and transfected until the cell coverage rate was 80%~90%. Transfection was performed using Lipofectamine TM 2000 transfection kit (invitrogen, V35113). The procedure was strictly carried out in a sterile environment according to the kit instructions.

The cells were cultured in 5%CO<sub>2</sub> incubator at 37°C for 48h, and the culture medium was changed every 6h.

#### **4. Blood and cell sample processing**

A 4ml of fasting peripheral venous blood was collected from the two groups, and placed at room temperature for 30min and then centrifuged for 10min (500×g) to obtain upper serum. Total Exosome Isolation Reagent (Thermo Fisher Scientific, 4478360) was added according to the ratio of 1: 5. The serum was mixed thoroughly by shaking and incubated for 30min (4°C). Then the blood was centrifuged for 10min (12000×g), the supernatant was discarded, and the sample was pipetted with PBS buffer. CD63 and CD81 were detected by Western blot, and exosomes were observed under microscope. Cell processing was the same as above. Total Exosome Isolation Reagent was from Thermo Fisher Scientific (4478559).

#### **5. PCR detection**

Total RNA in cells was extracted using TRIzol (Invitrogen, 15596018) and Total Exosome RNA & Protein Isolation Kit (Invitrogen, 4478545) was used to extract total RNA in exosomes. The purity and integrity of total RNA were detected by UV spectrophotometer and agarose gel electrophoresis, which required OD<sub>260</sub>/OD<sub>280</sub>>1.8 for qPCR detection. CDNA was prepared by reverse transcription (Beijing Tiangen Technology Co., Ltd., FP314), and PCR fluorescence quantification (Applied Biosystems, ABI PRISM 7000) was performed. The primer sequence was designed by Shanghai Lanxuan Biotechnology Co., Ltd. See Table 1. EasyScript One-Step RT-PCR SuperMix kit was purchased from Beijing Transgen Biotech (AE411-02). The test procedure was strictly carried out in accordance with the kit instructions, and a reaction system with a termination volume of 20μL was established. The thermal cycle parameter: 95°C, 5min. Reaction system: denaturation at 94°C for 30s, annealing at 60°C for 30s, for a total of 45 cycles.

#### **6. Western blot test**

The total protein was extracted by RIPA lysis method, the protein concentration was detected by BCA method and adjusted to 4μg/μL, and the protein was separated by 12% polyacrylamide gel electrophoresis. After separation, the membrane was transferred and incubated at 37°C for 1 hour. Then, it was placed in 5% defatted milk and sealed. SLU7, Caspase 9, E-cadherin, N-cadherin, Bax, Bcl-2, N-cadherin, β-actin primary antibody (1: 1000) were added and incubated overnight at 4°C. PBS was used for rinse for 3 times, goat anti-rabbit secondary antibody (1: 1000) was added, placed at room temperature for 1 hour, and washed with PBS for 3 times. ECL luminescent reagent was developed and fixed, and Quantity One software was used to carry out statistical analysis on the strip scanned by the film, and the relative protein expression level = gray value of the strip to be tested/gray value of β- actin in strip.

#### **7. CCK-8 detection**

Cells in each group were inoculated into 96-well plates with  $1 \times 10^6$  cells/well. Three multiple wells were set in each group. After 24, 48, 72, 96h the cells adhered to the wall, 10 $\mu$ L CCK-8 solution (Beijing Beyotime Biotechnology Co., Ltd., C0037), 0.5mg/mL, was added to each well. After incubation in 37°C incubator for 2 h, the optical density (OD) value at 450nm wavelength was measured by using an microplate reader, cell proliferation multiples were calculated, and growth curves were visualized.

## **8. Flow cytometry**

Cells to be tested in each group were collected, washed with PBS and then made into a suspension of  $1 \times 10^6$  cells/mL with binding buffer. Then Annexin V-FITC (Invitrogen, V35113) dye solution was added, fully shaken and mixed, and incubated at room temperature in dark for 10 min. PI dye solution was added and incubated at room temperature in dark for 5min. Flow cytometer (Beckman Coulter, CytoFLE S) was used for detection.

## **9. Transwell testing**

The cells of each treatment group were digested with pancreatin and inoculated into the 24-well plate of Transwell chamber. A 100 $\mu$ l (density:  $1 \times 10^6$  cells /ml) of cell suspension was added to the upper chamber, 250 $\mu$ L of medium containing 10% fetal bovine serum was added to the lower chamber, and cultured for 48h (37°C, 5%CO<sub>2</sub>). The chamber was taken out, cotton swabs were used to wipe off the cells in the upper chamber of microporous membrane, PBS was used to wash the chamber carefully, 4% paraformaldehyde was used to fix the chamber for 15min, 0.1% crystal violet solution was used to dye for 15 min, PBS was used to wash the chamber for 3 times, and the number of cells passing through the membrane was observed under the microscope.

## **10. Cell scratch test**

Cell suspension with a cell density of  $1 \times 10^6$  cells/ml was added to a 6-well plate and cultured overnight until monolayer cells were formed. A horizontal line was drawn on the monolayer cells with a pipette with a volume of 20 $\mu$ L and the exfoliated cells were washed with PBS. Under microscope, the scratch spacing was a. Subsequently, blood-free serum medium was added to continue culturing for 24 hours, and the scratches were examined under a microscope with a spacing of b. The cell mobility =  $(1-b)/a \times 100\%$ , 3 fields of view were randomly selected for examination, and the results were averaged.

## **11. Double luciferase report**

After human liver cancer cells were cultured to logarithmic growth phase, pmirGLO-RLU7-WT and pmirGLO-RLU7-MUT vectors were constructed and co-transfected into the cells with miR-1290-mimics and miR-NC respectively. After 48h of transfection, luciferase intensity was detected using double luciferase reporter.

## **Statistical method**

SPSS22.0 (Asia Analytics Formerly SPSS China) was used to analyze and process the data. The counting data was expressed in the form of (%), and chi-square test was used for comparison between groups. The measurement data were expressed in the form of (mean± standard deviation), and the comparison between groups adopted independent sample t test. One-way ANOVA and LSD back testing were used for comparison among groups. The comparison among multiple time points adopted repeated measurement analysis of variance and bonferroni back testing. The diagnostic predictive value was analyzed by ROC curve. The survival rate was calculated by Kaplan-Meier method, and the survival rate was compared by Log-rank test.  $P < 0.050$ , the difference was statistically significant.

## Result

### 1. Clinical significance of miR-1290 and SLU7 in liver cancer

Detection of exosomes protein markers CD63 and CD81 showed that the relative expression of CD63 and CD81 proteins in exosomes was significantly higher than that in tumor cells ( $P < 0.050$ ). Detection of miR-1290 and SLU7 mRNA expression levels in exosomes showed that miR-1290 in exosomes was significantly higher than that in tumor cells, and SLU7 mRNA was significantly lower than that in tumor cells ( $P < 0.050$ ). However, the relative expression of SLU7 protein in exosomes was also significantly lower than that in tumor cells ( $P < 0.050$ ). The relative expression of miR-1290 and SLU7 mRNA and protein in the peripheral blood of the two groups was detected, and it was found that the results were consistent with that in the exosomes ( $P < 0.050$ ). ROC curve analysis showed that when cut-off value was 3.295, miR-1290 had a sensitivity of 70.69%, specificity of 68.75%, AUC of 0.740 ( $P < 0.001$ ). However, when cut-off value was 2.855, the sensitivity, specificity and AUC of SLU7 mRNA for diagnosing hepatocarcinogenesis were 53.45%, 89.06% and 0.772 respectively ( $P < 0.001$ ). See Figure 1.

### 2. Relationship of miR-1290 with clinical pathology and prognosis of liver cancer

After analysis, it was found that miR-1290 was not related to the sex and age of liver cancer patients ( $P > 0.050$ ), but was closely related to tumor classification, staging, differentiation degree and metastasis ( $P < 0.001$ ). After 3 years follow-up for liver cancer patients, 59 patients were successfully followed up with a follow-up success rate of 92.19%. According to the cut-off value of miR-1290 for predicting the occurrence of liver cancer, the patients were divided into high miR-1290 group (miR-1290  $\geq$  3.295, n=26) and low miR-1290 group (miR-1290  $<$  3.295, n=33). Comparing the survival curves of the two groups for 3 years, the survival rate of low miR-1290 group was significantly higher than that of high miR-1290 group ( $P < 0.050$ ). See Table 2 and Figure 2.

### 3. Effect of exosome miR-1290 on hepatocellular carcinoma cells

The expression level of miR-1290 in exosomes of human hepatoma cells HepG2, Huh-7, BEL-7402, SMMC-7721 and human normal hepatocyte HL-7702 was detected, and miR-1290 was also found to be highly expressed in human hepatoma cells ( $P < 0.050$ ). The highest expression level was found in HepG2 and Huh-7 ( $P < 0.050$ ). Exosomes in HepG2 were extracted for subsequent experiments. The extracted

exosomes miR-1290 were co-cultured with BEL-7402 and SMMC-7721, and their biological behaviors were detected. The results showed that the cell proliferation, invasion, migration ability and Bcl-2, N-cadherin proteins in Exosomes miR-1290 group were significantly increased, while the apoptosis rate and SLU7, Caspase 9, E-cadherin, Bax proteins were significantly decreased ( $P < 0.050$ ). See Figure 3.

#### **4. Effect of SLU 7 on hepatocellular carcinoma cells**

The expression of SLU7 was inhibited, and the biological behavior of hepatoma cells was detected. The cell proliferation, invasion, migration ability and Bcl-2, N-cadherin proteins in SLU7-inhibitor group were significantly increased, while the apoptosis rate and Caspase 9, E-cadherin, Bax proteins were significantly decreased ( $P < 0.050$ ). See Figure 4.

#### **5. Relationship between miR-1290 and SLU7**

The same binding site between miR-1290 and SLU7 was found on the online website, and the targeting relationship between miR-1290 and SLU7 was further confirmed by double fluorescein reporter. After transfecting miR-1290-mimics, the relative expression of SLU7 protein was significantly decreased ( $P < 0.050$ ), while after transfecting miR-1290-inhibitor, the relative expression of SLU7 protein was significantly increased ( $P < 0.050$ ). See Figure 5.

#### **6. Rescue experiment**

The exosomes miR-1290 and over-expression vector SLU7 were added to BEL-7402 and SMMC-7721 cells, and the biological behaviors of the Exosomes miR-1290+SLU7-mimics group and the blank group were detected and found to have no difference in biological behaviors and relative protein expression ( $P > 0.050$ ). However, the cell proliferation, invasion, migration ability and Bcl-2, N-cadherin proteins in exosomemir-1290 group were significantly higher than those in the other two groups, while the apoptosis rate and SLU7, Caspase 9, E-cadherin, Bax proteins were significantly lower ( $P < 0.050$ ). See Figure 6.

## **Discussion**

Liver cancer, as a very common malignant tumor in clinical practice, has a high incidence rate and a poor prognosis [14]. At present, researchers at home and abroad are constantly trying to find a new diagnosis and treatment scheme for liver cancer, but no significant breakthrough has been made. miRNA is a hot spot in modern research, and its influence on tumor may be the breakthrough point for diagnosis and treatment of tumor diseases in the future [15-17]. Reviewing previous studies, we found that there have been many studies on the mechanism of the influence of miRNA on liver cancer [18,19], but studies on miRNA in exosomes are extremely rare. Exosomes are microvesicles containing complex RNA and proteins, with a size of about 30~150nm. All cell types can secrete exosomes under normal and pathological conditions [20]. It mainly comes from the multi-vesicular body formed by the invagination of intracellular lysosomal particles, and is released into the extracellular matrix after fusion of the outer membrane of the multi-vesicular body with the cell membrane [21]. At present, the research on the

mechanism of exosomes for diseases has just begun, and a thorough understanding of its functions may be of historic significance for the diagnosis and treatment of tumor diseases in the future.

This experiment analyzed the mechanism of miR-1290 in exosomes on liver cancer. The results showed that miR-1290 was highly expressed in liver cancer, while SLU7 was poorly expressed in liver cancer, suggesting that both might be involved in the occurrence and development of liver cancer. This was also consistent with the research of Kobayashi et al. [22] and Urtasun et al. [23], which could support our results. Further selecting HepG2 cells with the highest expression level of miR-1290 in liver cancer cells to extract exosomes miR-1290 was carried out for subsequent tests, it was found that the proliferation, invasion, migration and epithelial-mesenchymal transformation capabilities of liver cancer cells co-cultured with exosomes miR-1290 were significantly increased, while the apoptosis capability was significantly reduced, suggesting that miR-1290 plays a role of oncogene in liver cancer, while exosomes miR-1290 could enhance its tumor activity. Looking up relevant literature, we found that Yan et al. [24] were also studying the role of miR-1290 as a tumor-promoting gene in glioma, which also confirmed the consistency of the biological effects of miR-1290.

In addition, we found that the relative expression of SLU7 protein was significantly reduced in liver cancer cells co-cultured with exosome miR-1290. Therefore, the SLU7 protein was transfected into liver cancer cells after inhibition and its biological behavior was detected. It was found that the ability to inhibit the proliferation, invasion, migration and epithelial-mesenchymal transformation of SLU7 protein liver cancer cells was also significantly increased, while the ability to apoptosis was significantly decreased, suggesting that low expression of SLU7 protein in liver cancer also played a role in promoting tumor development. Some studies have claimed that miR-1290 promotes proliferation of colorectal cancer cells by targeting INPP4B [25], but the pathway of influence on liver cancer is still unclear. Through online website prediction, we found that miR-1290 and SLU7 had the same binding site, and double luciferase reporter enzyme confirmed that when miR-1290-mimics and PMIRGO-SLU7-WT co-transfected cells, luciferase activity was the lowest, and miR-1290 could inhibit the level of SLU7 by transfecting miR-1290 into liver cancer cells and detecting the relative expression of SLU7 protein, suggesting that miR-1290 participated in the occurrence and development of liver cancer by targeting SLU7. However, through rescue experiments, we found that the biological behavior of hepatoma cells co-cultured with exosome miR-1290 and SLU7 over-expression vector was not different from that of the blank group, and their proliferation, invasion, migration and epithelial-mesenchymal transformation capabilities were significantly lower than those of hepatoma cells co-cultured with exosome miR-1290, suggesting that exosome miR-1290 accelerated the proliferation, invasion, migration and epithelial-mesenchymal transformation capabilities of hepatoma through targeting inhibition of SLU7 and reduced its apoptosis capability.

This experiment preliminarily discussed that exosome miR-1290 participated in the occurrence and development of liver cancer through targeting inhibition of SLU7. However, due to the limited experimental conditions, there are still deficiencies. For example, we have not analyzed the signaling pathway of exosome miR-1290 targeted SLU7 in regulating liver cancer, and we have not conducted nude

mouse tumorigenesis experiment for further verification. Moreover, more in-depth experimental analysis is still needed on miR-1290 as a therapeutic target for liver cancer in the future. We will carry out more experimental analysis to supplement the above deficiencies and obtain the best experimental results.

To sum up, exosome miR-1290 may be a potential target for the diagnosis and treatment of liver cancer in the future by targeting inhibition of SLU7 in the occurrence and development of liver cancer.

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

### **Availability of data and materials**

The authors confirm that the data supporting the findings of this study are available within the article.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

Fuyong Li and Tingyong Fan conducted the experiments; Hao Zhang and Yumin Sun designed the experiments and wrote the paper.

### **Acknowledgements**

Not applicable

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## Tables

Table 1: Primer sequence

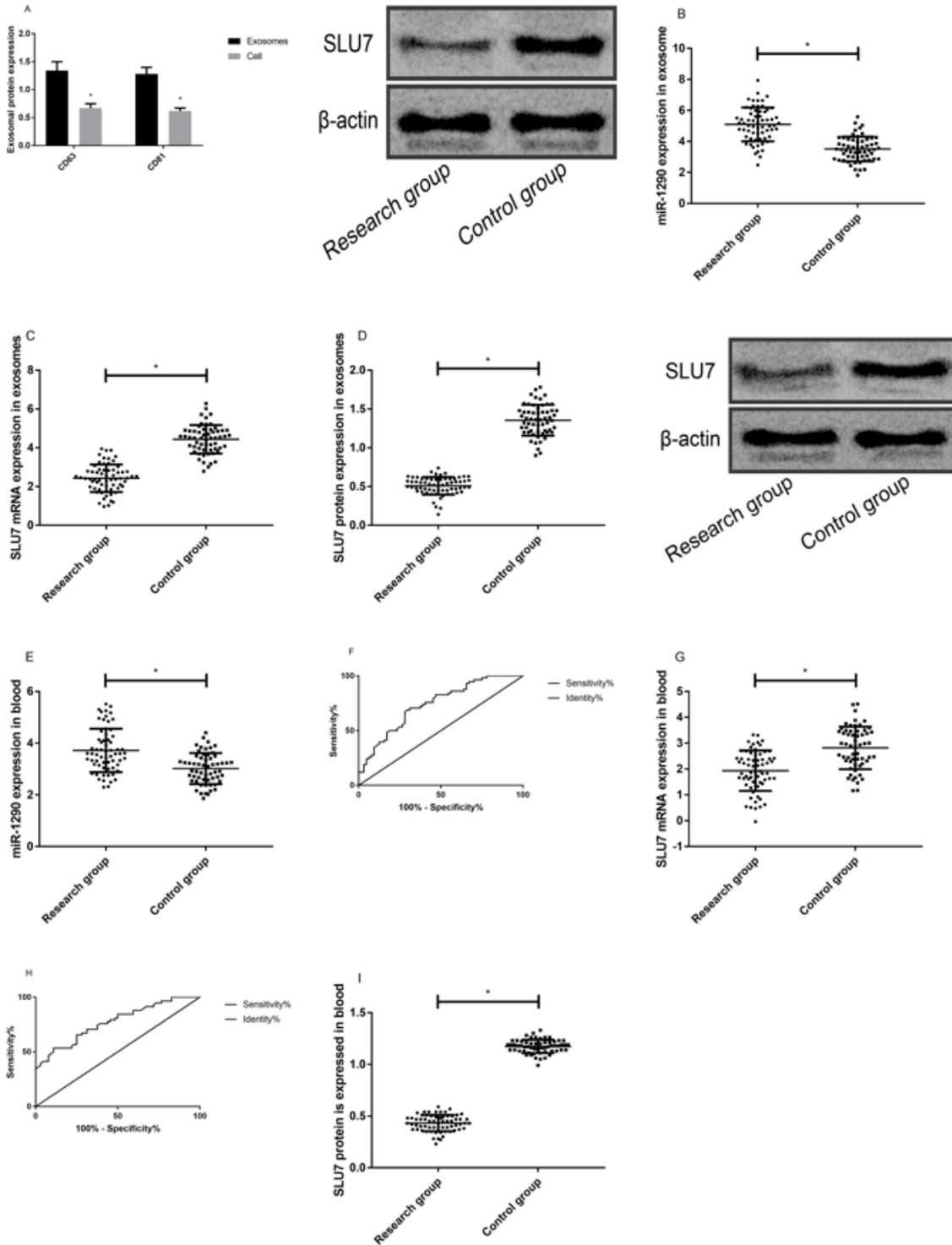
	F 5'-3'	R 5'-3'
miR-1290	ACACTCCAGCTGGGTGGATTTTGG	CTCAACTGGTGTCTGCGTGA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
SLU7	AGTTAGTGCACACCCATGAC	CAGGAGCATTGCCAGTTT
GAPDH	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG

**Table 2: Relationship between miR-1290 and clinicopathology of hepatocellular carcinoma**

Pathological features	n	miR-1290	t	P
Sex			0.876	0.384
Male	42	3.80±0.38		
Female	22	3.71±0.41		
Age			0.529	0.599
≤ 50 years old	15	3.78±0.40		
> 50 years old	49	3.72±0.38		
Tumor typing			22.810	0.001
Simple type	24	3.48±0.34		
Hardened type	19	3.75±0.28*		
Inflammatory type	21	4.22±0.46*#		
Staging of liver cancer			55.410	0.001
Phase I	16	3.20±0.18		
Phase II	29	3.64±0.32@		
Phase III	19	4.18±0.27@&		
Degree of differentiation			7.054	0.001
Medium and high	42	3.52±0.28		
Low	22	4.08±0.34		
Transferred			8.213	0.001
Yes	15	3.41±0.34		
No	49	4.16±0.30		

Note: \* indicates that compared with simple type,  $P < 0.050$ ; # indicates that compared with hardened type,  $P < 0.050$ ; @ indicates that compared with phase I,  $P < 0.050$ ; & indicates that compared with phase II,  $P < 0.050$ .

## Figures



**Figure 1**

Clinical significance of miR-1290 and SLU7 in liver cancer. A) the relative expression of CD63 and CD81 proteins in exosomes and immunoblotting figure. B) the expression level of miR-1290 in exosomes. C) the expression level of SLU7 mRNA in exosomes. D) the relative expression of SLU7 protein in exosomes and immunoblotting figure. E) miR-1290 expression level in peripheral blood of the study group and the control group. F) ROC curve of miR-1290 in peripheral blood for predicting hepatocarcinogenesis. G) the

expression level of SLU7 mRNA in peripheral blood of study group and control group. H) ROC curve of H)SLU7 mRNA for predicting hepatocarcinogenesis. I) the relative expression of SLU7 protein in the peripheral blood of the study group and the control group and the immunoblotting figure.

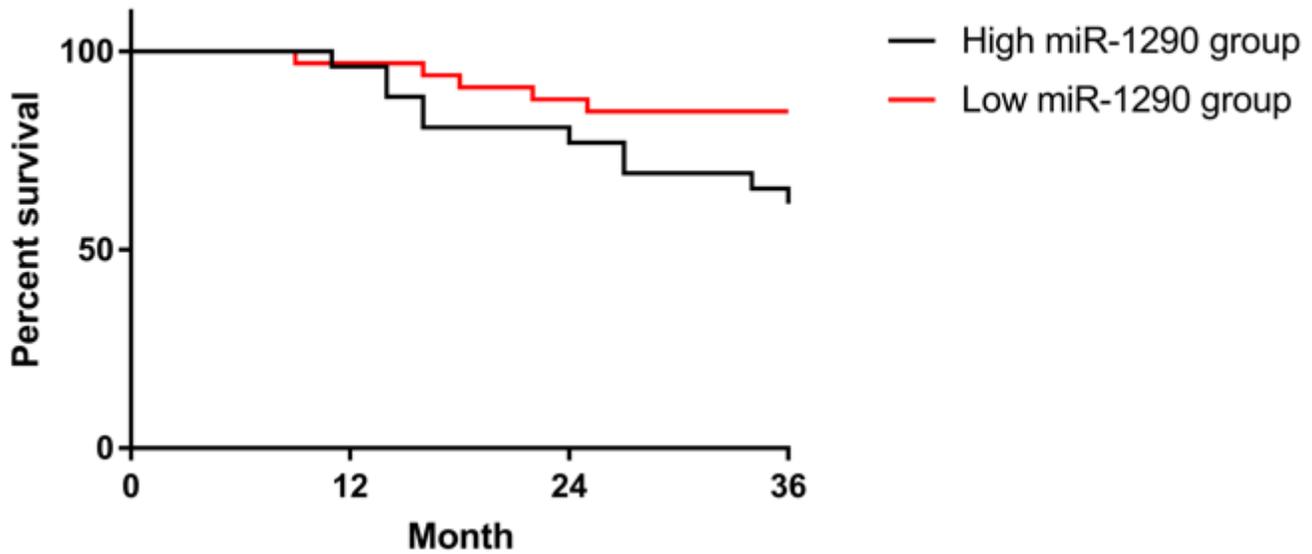
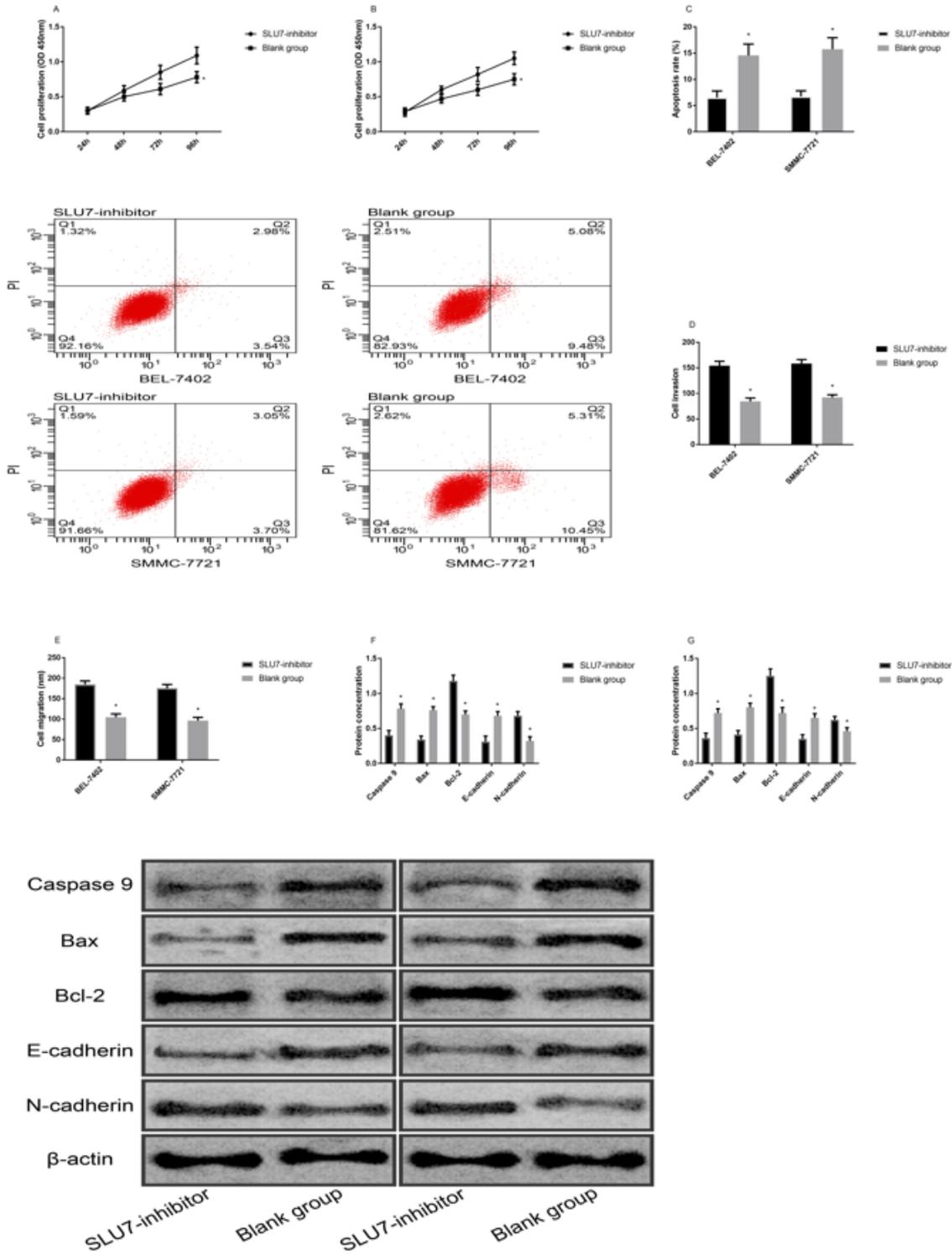


Figure 2

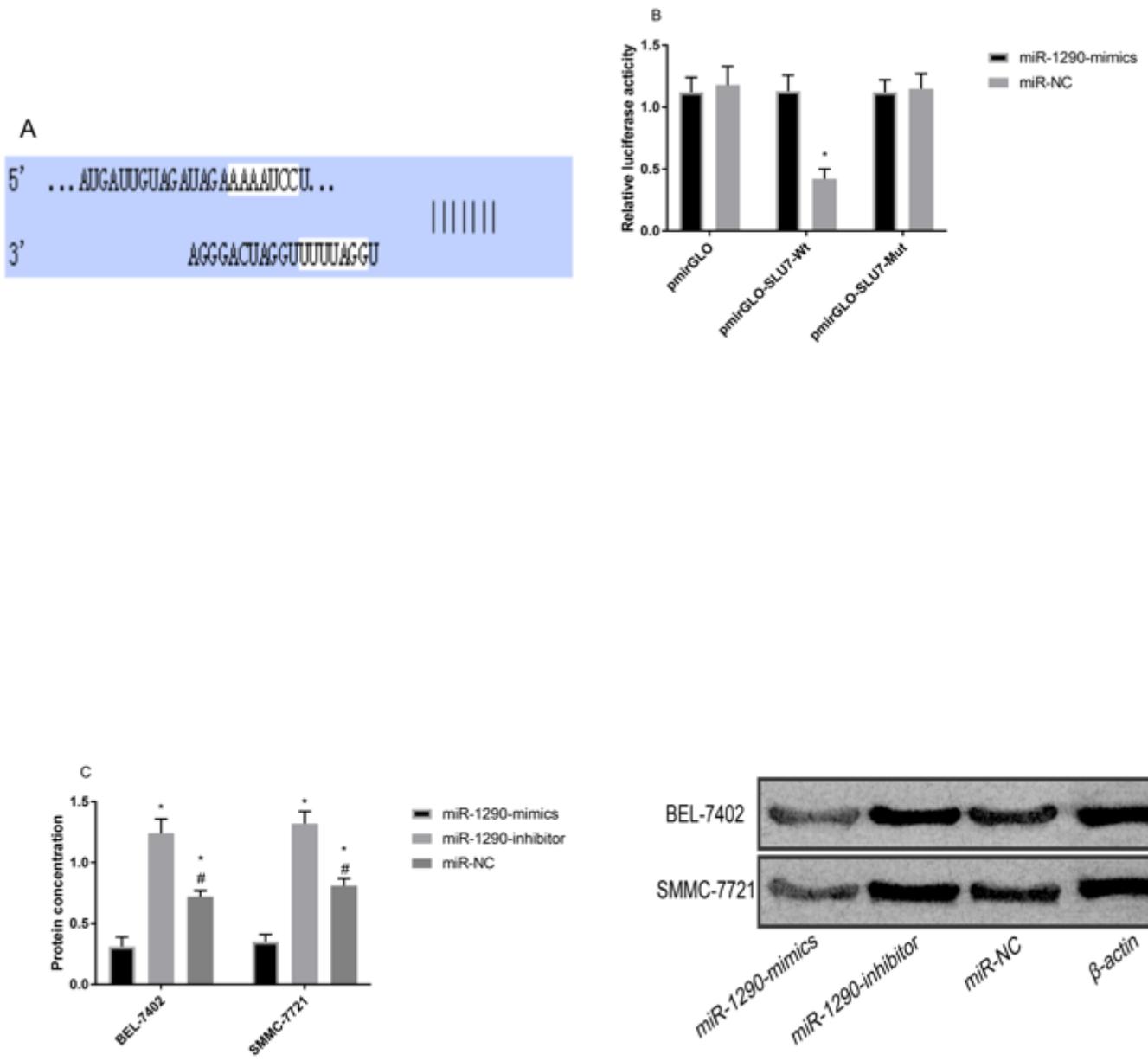
Three-year survival curve of prognosis between high miR-1290 group and low miR-1290 group.





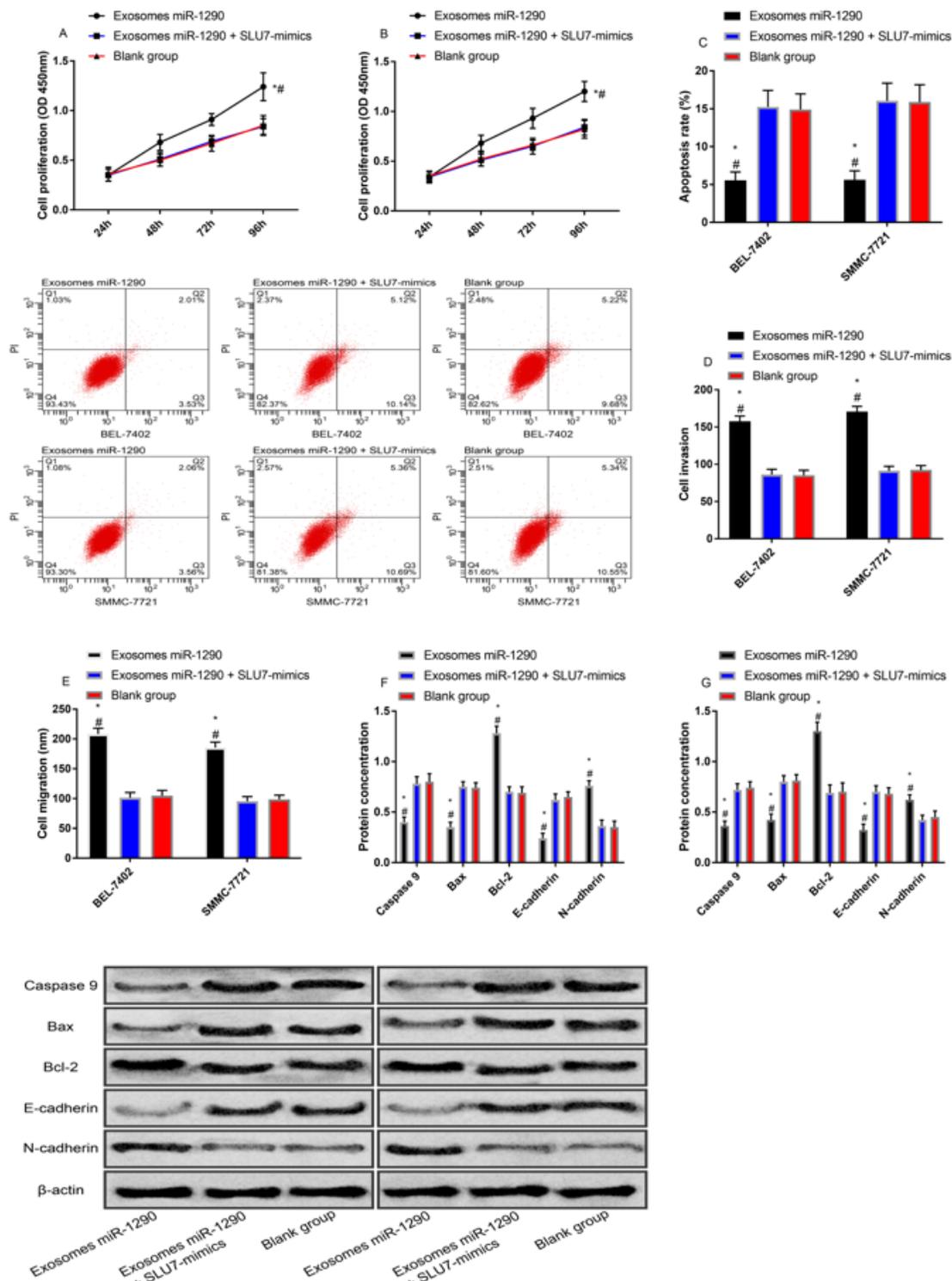
**Figure 4**

Effect of SLU 7 on hepatoma cells. A) the proliferation of BEL-7402 cells. B) the proliferation of SMMC-7721 cells. C) the apoptosis rate and flow cytometry count. D) cell invasion. E) cell migration. F) the relative expression of proteins in BEL-7402 cells and immunoblotting figure. G) the relative expression of proteins in SMMC-7721 cells and immunoblotting figure.



**Figure 5**

Relationship between miR-1290 and SLU7. A) the online websites predicted that miR-1290 and SLU7 have the same binding site. B) double fluorescein reporter enzyme verified the relationship between miR-1290 and SLU7. C) the relative expression of SLU7 protein and immunoblotting figure after transfection of miR-1290 into hepatoma cells.



**Figure 6**

Rescue experiment. A) the proliferation of BEL-7402 cells. B) the proliferation of SMMC-7721 cells. C) the apoptosis rate and flow cytometry count. D) cell invasion. E) cell migration. F) the relative expression of proteins in BEL-7402 cells and immunoblotting figure. G) the relative expression of proteins in SMMC-7721 cells and immunoblotting figure.